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Evaluation of polymorphisms in inflammatory mediator and cellular adhesion genes as risk factors for feline infectious peritonitis

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Abstract

Objectives

Feline infectious peritonitis (FIP) is a high mortality infectious disease. Single nucleotide polymorphisms (SNPs) in the genes encoding interferon gamma (*IFNG*), tumour necrosis factor alpha (*TNFA*) and DC-SIGN (*CD209*) have been associated with increased and decreased risk of developing FIP. This study was designed to determine whether these associations were present in a UK population of pedigree cats using samples from cats euthanased with a confirmed diagnosis (FIP, n=22; non-FIP, n=10) or clinically healthy cats over 11 years of age (n=3).

Methods

DNA was extracted from tissue (n=32) or blood (n=3) and PCR performed for regions of *IFNG*, *TNFA* and *CD209*. PCR amplicons were sequenced, each SNP genotype determined, and genotype/allele frequency for each SNP and FIP status compared.

Results

No significant association was found between genotype and FIP status for any SNP analysed. There was a trend for the heterozygous CT genotype at both *IFNG* g.401 and g.408 to be associated with FIP (P=0.13), but this genotype was also found in a substantial proportion of non-FIP cats. There was also a trend for the heterozygous CT genotype at *IFNG* g.428 to be associated with FIP (P=0.06), although most cats with FIP had the CC genotype at this locus. No associations were found between any allele at *TNFA* g.-421, *CD209* g.1900, *CD209* g.2276, *CD209* g.2392 and *CD209* g.2713 and FIP.

Conclusions and relevance

The use of the *IFNG*, *TNFA* and *CD209* SNPs described to predict risk of FIP cannot currently be recommended.

Introduction

Feline infectious peritonitis (FIP) is a high mortality infectious disease of cats caused by feline coronavirus (FCoV). The prevalence of infection with FCoV can reach 90% in multi-cat households ¹, but FIP develops in less than 5% of infected cats ². Feline genetic factors are suspected of playing a role in the development of FIP with studies from North America and Australia showing an increased risk of FIP in pedigree over non-pedigree cats ³⁻⁶. In addition, some breeds ^{3, 4, 6}, and even bloodlines within a breed ⁷, appear to have a higher risk of developing FIP than others. There also appears to be some variation in breed predisposition between continents ^{3, 4, 6}. Abyssinians and Rexes were found to be at high risk and Persians at low risk in both the USA and Australia. However, Burmese were found to be at high risk in Australia and at low risk in the USA, while Himalayans (colour-point Persians) were found to be at high risk in the USA and at low risk in Australia. A German study found no association between breed and predisposition to FIP, but the number of pedigree cats included was small and mixed breed and Domestic shorthaired cats were amalgamated in one group ⁸.

During the pathogenesis of FIP, following infection of enterocytes, FCoVs infects monocytes and macrophages where they replicate and are systemically spread ⁹. Entry of FCoVs into some feline cells is mediated by interactions between the viral spike glycoprotein and host receptor proteins, some of which have yet to be elucidated ¹⁰. Dendritic cell-specific intercellular adhesion molecule-grabbing non-integrin (DC-SIGN), a mannose-binding lectin, has been shown to be a key co-receptor during monocyte entry of both serotype I and II FCoVs ¹¹. An aberrant immune-response to the presence of FCoV in macrophages and monocytes results in the development of vasculitis and tissue granulomas, which in turn leads to the body cavity effusions and mass lesions that characterise this disease ¹².

Studies have suggested that decreased risk of developing FIP is associated with a strong Th1 cell-mediated immunity, with higher concentrations of interferon-gamma (IFN- γ) mRNA or protein in the blood of healthy FCoV-infected cats compared with cats with FIP^{13, 14}. Interestingly, one of these studies also showed high levels of IFN- γ in the abdominal effusion of cats with FIP, despite having low levels in the blood, suggesting that cell-mediated immunity may also play a role in mediating pathogenesis¹⁴. A vaccination study indicated that susceptibility to FIP also likely involves dysregulation of the IFN- γ /tumour necrosis factor-alpha (TNF- α) response, with production of high IFN- γ and low TNF- α levels associated with decreased risk, and production of low IFN- γ and high TNF- α levels associated with increased risk¹⁵. Another study also showed that transcription of inflammatory cytokines and chemokines, including TNF- α and IFN- γ , was upregulated in the mesenteric lymph nodes of cats with FIP, consistent with inflammatory pathway activation¹⁶.

Feline single nucleotide polymorphisms (SNPs) have been associated with risk of developing FIP. One study that looked at the IFN- γ gene (*IFNG*) found the CT heterozygous genotype (CT) at both *IFNG* g.401 and *IFNG* g.408 to be associated with increased risk of FIP¹⁷. These two SNPs appeared to be in complete linkage disequilibrium, i.e. the genotypes at these two loci were identical in all cats examined. In addition, the presence of a T allele at *IFNG* g.428 was associated with decreased risk of FIP. Another study that looked at the TNF- α gene (*TNFA*) found the presence of a T allele at *TNFA* g.-421 to be associated with decreased risk of FIP¹⁸. In addition, when the DC-SIGN gene (*CD209*) was examined, the presence of an A allele at *CD209* g.1900 or a T allele at *CD209* g.2713 were reported to be associated with increased risk of FIP, whilst

presence of a T allele at *CD209* g.2276 or an A allele at *CD209* g.2392 were found to be associated with decreased risk of FIP ¹⁸.

The aim of the present study was to evaluate the utility of these SNPs in predicting the risk of FIP in a cohort of pedigree cats using samples from the Bristol FIP Biobank.

Materials and Methods

Animals and samples

The cats in the study comprised those of known pedigree within the Bristol FIP Biobank (n=32) for which a definitive diagnosis of either FIP (n=22) or a disease other than FIP (n=10) were available, and elderly (>11 year), staff-owned pedigree cats (n=3) that were considered to be healthy based on history, clinical examination and blood testing. The FIP cats comprised British Shorthair (n=6), Ragdoll (n=6), Birman (n=3), Maine Coon (n=2), Siamese (n=2) and one each of Abyssinian, British Blue and Burmese. The non-FIP cats comprised Bengal (n=3), Ragdoll (n=2) and one each of Birman, British Shorthair, Devon Rex, Havana, Maine Coon, Persian-cross, Siamese and Tiffanie.

For the cats from the Bristol FIP Biobank, tissue samples were collected at post-mortem examination. Some of these cats had been included in earlier studies ^{16, 19-21}. Formalin-fixed tissue samples were subjected to histological examination, including immunohistochemistry for FCoV antigen to confirm FIP, and categorised as FIP or non-FIP as previously described ¹⁹. Tissue samples collected into RNeasy® (Life Technologies, ThermoFisher Scientific), faeces and body cavity fluid were stored at -80°C prior to processing. For the staff-owned cats, excess blood from samples collected for routine health screening was stored at -80°C prior to processing.

DNA extraction, amplification and sequencing

Feline genomic DNA was extracted from stored samples as previously described for total nucleic acids ¹⁹. Feline genomic DNA was extracted from blood (100 µl) using a Chemagic 360 automated platform (Perkin-Elmer) in combination with the Chemagic body fluids nucleic acid kit (Perkin-Elmer) and eluted in elution buffer (100 µl).

Tissue, faeces and body cavity fluid samples from the Bristol FIP Biobank were subjected to FCoV quantitative reverse-transcriptase PCR, with results previously reported elsewhere ¹⁹.

PCRs to amplify gene fragments containing the target SNPs were performed using 2x GoTaq Master Mix (Promega), 200nM forward and reverse amplification primers (see **Table 1**) and 5 µl DNA in a total volume of 25 µl. Thermal cycling was performed in a PTC-200 DNA Engine (MJ Research) with the following thermal profile for *IFNG* and *TNFA*: 95°C for 2 min followed by 40 cycles of 95°C for 20 s, 60°C for 20 s and 72°C for 20 s; and the following thermal profile for *CD209*: 95°C for 2 min followed by 35 cycles of 95°C for 20 s, 64°C for 20 s and 72°C for 60 s. Amplicons were visualised by agarose gel (1% w/v) electrophoresis, using ethidium bromide stain and viewed using a GelDoc-It® Imaging System (UVP LLC, Cambridge, UK).

Amplicons (*IFNG* 559bp (primer set 1) or 799bp (primer set 2), *TNFA* 498bp, *CD209* 1095bp) were purified (NucleoSpin® Extract II, Macherey-Nagel, Germany) according to the manufacturer's instructions and sequenced (DNA Sequencing & Services, University of Dundee, Dundee, UK www.dnaseq.co.uk) (see **Table 1** for primer sequences). The *IFNG* amplicon was sequenced using the forward and reverse amplification primers. The *TNFA* amplicon was

sequenced using a separate forward sequencing primer and the reverse amplification primer. The *CD209* amplicon was sequenced using separate forward sequencing primers and the reverse amplification primer. The derived sequences were assembled using MacVector v15.5.4 (MacVector Inc, Cambridge, UK). Since allele dropout was seen in some cats for *IFNG* using primer set 1, *IFNG* primer set 2 was used in these cats.

Data Analysis

SNPs were described relative to their genomic position from the 'A' of the start codon of the relevant gene. The association between each genotype at each position and FIP status (FIP vs. non-FIP; non-FIP comprised cats that were euthanased for diseases other than FIP, and the healthy alive cats) was analysed using Fisher's exact test with the Freeman-Halton extension. The association between each allele frequency at each position and FIP status was analysed using Fisher's exact test. Analyses were performed in XLSTAT v2017.6 (Addinsoft, NY, USA). A P value of ≤ 0.05 was considered statistically significant.

Results

Data for each sample (including FIP status and genotype for each SNP) are presented in the **supplementary table**. All cats with FIP had FCoV detectable in one or more tissue samples by PCR, and by definition at least one tissue was positive by immunohistochemistry for FCoV antigen within inflammatory lesions. Three of the deceased non-FIP cats were shedding FCoV in faeces at time of death and two had FCoV detected by PCR in tissue samples collected post-mortem (immunohistochemistry was negative for FCoV antigen in these tissues); the remaining non-FIP cats were negative for FCoV by PCR in all samples available for testing. FCoV serology was not

available for cats in the Bristol FIP Biobank. One of the living cats was reported to be seronegative for FCoV at 12 years of age.

***IFNG* SNPs**

The *IFNG* g.401 and g.408 SNPs were in complete linkage disequilibrium, with identical genotypes at these loci in all 35 cats. **Table 2** shows the number and percentage of cats with each genotype at these loci. No genotype was found to be associated with FIP at either locus ($P=0.13$), although the percentage of cats with the heterozygous CT genotype at both loci was 2.5 times greater in FIP cats (13/22; 59.1%) than in non-FIP cats (3/13; 23.1%). Neither the C nor T allele at either locus was associated with FIP status.

All 13 non-FIP cats had genotype CC at *IFNG* g.428 whereas only 16/22 (72.7%) of FIP cats had this genotype. No genotype was found to be associated with FIP status at this locus ($P=0.06$), although no cats in the non-FIP group had the heterozygous CT genotype and no cats in either group had the TT genotype. Neither the C nor T allele at either locus was associated with FIP status.

SNPs were also found (see the **supplementary table**) at *IFNG* g.230, *IFNG* g.253, *IFNG* g.308, *IFNG* g.333, *IFNG* g.468, *IFNG* g.523 and *IFNG* g.524 but none were associated with FIP status.

***TNFA* SNPs**

No genotype at *TNFA* g.-421 was associated with FIP status, and neither were the C or T alleles (**Table 3**).

CD209 SNPs

All 35 cats had genotype GG at locus *CD209* g.1900. At locus *CD209* g.2276, 34 cats had genotype CC and one FIP cat had genotype CT. At locus *CD209* g.2392 (**Table 4**), the majority of FIP and non-FIP cats had genotype GG, and there was no association between genotype, nor between G or A alleles, and FIP status. At locus *CD209* g.2713 (**Table 4**), the majority of FIP and non-FIP cats had genotype CC, and there was no association between genotype, nor between C or T alleles, and FIP status. A further SNP was found at *CD209* g.2718 (see the **supplementary table**) but was not associated with FIP status.

Discussion

A SNP describes nucleotide variation at a single point within the genome. SNPs within protein coding regions of a gene (i.e. exons) may change the translated amino acid sequence or result in premature termination of translation and protein truncation. SNPs in non-coding regions (e.g. introns) may increase or decrease transcription factor binding or change gene splicing and result in altered protein levels or alternative isoforms. Single nucleotide polymorphisms in genes such as *IFNG*, *TNFA* and *CD209* can significantly change the amount and function of these important immunomodulatory proteins and potentially influence disease risk ²²⁻²⁵. A number of SNPs in these genes have been reported to be associated with increased or decreased likelihood of FIP ^{17, 18}. These comprise: three SNPs in non-coding regions of *IFNG* (intron 1 *IFNG* g.401, g.408, and *IFNG* g.428); one SNP within the non-coding gene promotor region of *TNFA* (*TNFA* g.-421); one SNP within the extracellular domain of the protein (*CD209* g.1900) and three SNPs in non-coding regions (intron 6 *CD209* g.2276 and *CD209* g.2392; intron 7 *CD209* g.2713). Out of these nine SNPs only one (*CD209* g.1900) is predicted to affect protein structure/function by substituting Trp128 for a stop codon. The previously reported intronic SNPs found associated with disease

status may be in linkage disequilibrium with other loci that have yet to be discovered, and it is these loci that confer disease susceptibility or resistance. Our study aimed to characterise these previously reported SNPs in a pedigree population of 35 UK cats, and compare genotype and SNP allele frequency to FIP status.

Our results did support the previously reported finding of complete linkage disequilibrium between *IFNG* g.401 and *IFNG* g.408 ¹⁷, as this was present in all cats tested. However, a significant association between the heterozygous CT genotypes at *IFNG* g.401 and g.408 and FIP status was not found in this study, although a previous study had found significance with 62.1% (n=18/29) of FIP cats having the CT genotypes as compared to 31.7% (n=26/82) of non-FIP cats (Fisher Exact; 0.004) ¹⁷. In our study there was, nonetheless, a trend for the CT genotypes to be more common in FIP cats (59.1%) compared with non-FIP cats (23.1%). Our study was limited by the number of pedigree cats that had been euthanised for a non-FIP cause, and it is possible that, with the greater power of a larger study, a significant association of the CT genotypes with FIP could have been found. However, it should be noted that in both this and the previously published study ¹⁷, a significant number of non-FIP cats had the CT genotypes and absence of the CT genotypes was not a good predictor that cats will not develop FIP. Therefore, at this time the selection of breeding cats based on the presence or absence of the CT genotypes at *IFNG* g.401 and *IFNG* g.408 cannot be recommended.

There was also a trend for the heterozygous CT genotype at *IFNG* g.428 to be associated with FIP (P=0.06), although most cats with FIP and all of the non-FIP cats had the CC genotype at this locus. This is in contrast to previously reported findings, which showed the T allele at *IFNG* g.428 to be more prevalent in the non-FIP group ¹⁷. Indeed, in our study the T allele at *IFNG* g.428

was only found in FIP affected cats (n=6/22). The difference in association between the two studies might be explained by different genetic populations.

Our results found no evidence of any association between FIP risk and SNPs *TNFA* g.-421, *CD209* g.1900, *CD209* g.2276, *CD209* g.2392 and *CD209* g.2713. These SNPs were previously reported to confer either increased (G>A at *CD209* g.1900, odds ratio (OR) 3.7; C>T at *CD209* g.2713, OR 1.75) or decreased (C>T at *TNFA* g.-421 OR 3.9; C>T at *CD209* g.2276, OR reported as not available; G>A at *CD209* g.2392, OR 2.6) risk of developing FIP ¹⁸. In addition, these authors described a G>A transition at *CD209* g.1900 that was not seen in our study population ¹⁸.

We are aware that the current study has several limitations; these comprise the number of cats included, the 'potential' for the non-FIP cats to have developed FIP at a later stage, and the possibility for genetic variation between breeds. However, all three of these limitations were also present in the other studies ^{17, 18}. We had a total of 35 cats of which 13 were classified as non-FIP and 22 as FIP. Using the genotype frequency determined for the population described in our study at least 28 cats would be required in each group to achieve statistical significance between the groups. However, we were limited by the number of pedigree cat samples that were available in the Bristol FIP Biobank. The possibility that the deceased cats in the non-FIP group could have gone on to develop FIP, were they not to have developed the alternative disease for which they were euthanased, cannot be ruled out. The non-FIP cats used in previous studies were all less than 3 years of age at time of enrolment with evidence of FCoV infection (positive by FCoV PCR on one or more of blood, nasal/ oral/ conjunctival/ rectal swabs, and/ or faeces) ^{17, 18}, and were alive at least 2 years following recruitment in one of these studies ¹⁷, whereas in the current study,

the non-FIP cats had either been euthanased for reasons other than FIP with a median age of 5.8 years (range 4 month-13.8 years), or were alive (≥ 11.8 years, as of November 2018). Data regarding prior exposure, infection and shedding status were available for a proportion of the non-FIP cats (see **Supplementary data 1**), but not for all, so did not provide any meaningful results. One of the living cats with CT genotype at both *IFNG* g.401 and g.408 had a negative FCoV serology result; however, it had resided in a single cat household for nearly its entire life after being obtained from a multicat household as a kitten, such that early prior exposure could not be ruled out. FCoV serology was not available for the other two living cats. It cannot be ruled out that some pedigree breeds may be fixed for a single allele for a specific SNP, potentially creating bias; however, for the most frequently represented breeds (British Shorthair; Ragdoll; Birman) different genotypes were present at the target loci. It was not possible to statistically compare results within individual breeds due to the small numbers in each group. In the study by Hsieh and Chueh ¹⁷, approximately half of the cats were reported to be of known pedigree, or pedigree cross; however, pedigree breed and SNP status data, including any relationship between the two, were not reported. Future studies could focus on individual breeds for which risk of developing FIP appears to be greater (e.g. Abyssinians and Rexes), to exclude interbreed variation.

Conclusion

While not achieving statistical significance, the results of our study support a possible weak positive association between the CT genotypes at *IFNG* g.401/g.408 and risk of developing FIP. However, as the CT genotypes at these positions were present in a significant proportion of non-FIP cats (3/13) and absent in a significant proportion of FIP cats (9/22), a genetic test based on these two SNPs alone to predict risk of developing FIP, or to guide breeding programmes to reduce the risk of FIP, cannot be recommended at this time.

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Conflict of Interest

The authors have no conflicts of interest

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Ethical Approval

The project has been approved under ethical review by the University of Bristol Animal Welfare and Ethical Review Board (VIN/14/013; VIN/16/020).

Informed Consent

This was a retrospective study of samples previously collected with full informed consent from owners who agreed that samples could be used for research purposes.

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Table 1. Primers used in PCR amplification (A) and sequencing (S) for each gene

Gene	Primer	Use	Sequence
Interferon- γ	Forward 1	A / S	5'-ATTTTCGCTTTCCAGCTTTG-3'
	Reverse 1	A / S	5'-TTTAAGCCCGAACCTAAGGAC-3'
	Forward 2	A / S	5'-TTGTCTGCTGGTCGGCTATT-3'
	Reverse 2	A / S	5'-CTCCTACAAAATCTCAGCGAGTG-3'
Tumour necrosis factor- α	Forward	A	5'-CCACCAAGGACTCTGCTTTC-3'
	Forward	S	5'-AAAAAATGGAGGGAATTGGG-3'
	Reverse	A / S	5'-GGGGGTCTGGAGTTGCTT-3'
DC-SIGN	Forward	A	5'-CTATGGCTCCTGGCTTCCT-3'
	Forward 1	S	5'-TTGGGAAGGTCATGTGAGAGA-3'
	Forward 2	S	5'-CCTGGGGACTGAGCTGAC-3'
	Reverse	A / S	5'-GAGGGTTCAAACACCACTCC-3'

Table 2. Interferon- γ gene (*IFNG*) genotype and allele frequency results for non-FIP and FIP cats included in the study. *P*-value indicates likelihood that a genotype or allele is associated with FIP status as determined by Fisher's exact test (with the Freeman-Halton extension as required).

SNP		Number of non-FIP cats (%)	Number of FIP cats (%)	<i>P</i> value
<i>IFNG</i> g.401	CC	5 (38.5)	5 (22.7)	0.13
	CT	3 (23.1)	13 (59.1)	
	TT	5 (38.5)	4 (18.2)	
	Allele C	13 (50)	23 (52.3)	1.0
	Allele T	13 (50)	21 (47.2)	
<i>IFNG</i> g.408	CC	5 (38.5)	5 (22.7)	0.13
	CT	3 (23.1)	13 (59.1)	
	TT	5 (38.5)	4 (18.2)	
	Allele C	13 (50)	23 (52.3)	1.0
	Allele T	13 (50)	21 (47.2)	
<i>IFNG</i> g.428	CC	13 (100)	16 (72.7)	0.06
	CT	0 (0.0)	6 (27.3)	
	TT	0 (0.0)	0 (0.0)	
	Allele C	26 (100)	38 (86.4)	0.08
	Allele T	0 (0.0)	6 (13.6)	

Table 3. Tumour necrosis factor- α gene (*TNFA*) genotype and allele frequency results for non-FIP and FIP cats included in the study. *P*-value indicates likelihood that a genotype or allele is associated with FIP status as determined by Fisher's exact test (with the Freeman-Halton extension as required).

SNP		Number of non-FIP cats (%)	Number of FIP cats (%)	<i>P</i> value
<i>TNFA</i> g.-421	CC	12 (92.3)	20 (90.9)	1.0
	CT	1 (7.7)	2 (9.1)	
	TT	0 (0.0)	0 (0.0)	
	Allele C	25 (96.2)	42 (95.5)	1.0
	Allele T	1 (3.8)	2 (4.5)	

Table 4. DC-SIGN gene (*CD209*) genotype and allele frequency results for non-FIP and FIP cats included in the study. *P*-value indicates likelihood that a genotype or allele is associated with FIP status as determined by Fisher's exact test (with the Freeman-Halton extension as required).

SNP		Number of non-FIP cats (%)	Number of FIP cats (%)	<i>P</i> value
<i>CD209</i> g.2392	GG	7 (53.8)	16 (72.7)	1.0
	GA	5 (38.5)	5 (22.7)	
	AA	1 (7.7)	1 (4.6)	
	Allele G	19 (73.1)	37 (84.1)	0.36
	Allele A	7 (26.9)	7 (15.9)	
<i>CD209</i> g.2713	CC	10 (76.9)	14 (63.6)	0.81
	CT	3 (23.1)	7 (31.8)	
	TT	0 (0.0)	1 (4.6)	
	Allele C	23 (88.5)	35 (79.5)	0.51
	Allele T	3 (11.5)	9 (20.5)	

Legends:

Supplementary table: Data for each sample, including FIP status, breed, age at sampling, cause of death (or alive at time of writing), and genotype for each SNP tested